Novel Tools for High-Throughput Differentiation of *Bacillus* strains using Infrared Microspectroscopy.

E.M. Grasso, A.E. Yousef, and L.E. Rodriguez-Saona

ABSTRACT

Bacillus spores are resistant to processing and sanitation procedures, making their control of considerable importance in the food industry to prevent product spoilage and foodborne diseases. The objective of this study was to develop a rapid method for the differentiation, identification, and classification of *Bacillus* species at the strain level using infrared microspectroscopy and multivariate pattern-recognition techniques. Vegetative cells (10^4 CFU/mL) of *Bacillus* spp., including *Bacillus cereus* (4), *B. mycoides* (3), and *B. thuringienisis* (3), were filtered onto hydrophobic-grid membranes (HGM). The HGM were placed on Tryptic Soy Agar (TSA) and incubated at 42°C for 18-20h. HGM were removed from the agar, dried, and biomass of individual strains were measured by Attenuated Total Reflectance (ATR) IR microspectroscopy. Soft Independent Modeling of Class Analogy (SIMCA) models, generated from transformed spectra in the 1100-900 cm^{-1} region, exhibited clusters that permitted accurate strain-level classification of all isolates with zero-misclassifications. Major discrimination was related to signal from lipids likely present in the cellular membrane. Interclass distances, a measure of the distance between clusters based on factor loadings, provided further information regarding separation of classes. In conclusion, a simple IR microspectroscopy technique combined with multivariate analysis could provide the food industry with a rapid and reagent-free screening procedure to complement elaborate molecular identification methods.

INTRODUCTION

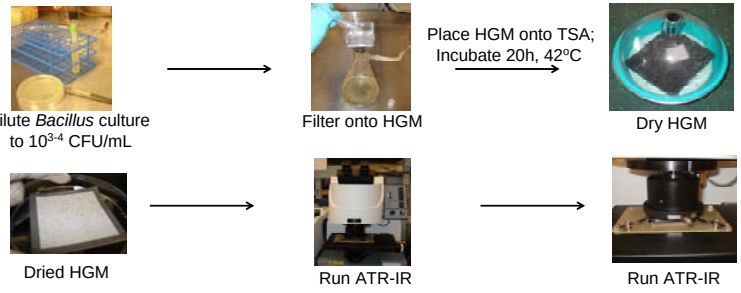
The ubiquity of the spore-forming *Bacillus* genus is a threat to the safety of a wide range of foods, including dairy products, grains, spices, and ready-to-eat meals[1,2]. *Bacillus* spores are resistant to processing and sanitation procedures, making their control of considerable importance in the food industry to prevent product spoilage and food-borne diseases[2]. Pathogenecity in the genus *Bacillus* has been mainly associated to bacterial strains belonging to the *B. cereus* and *B. anthracis* species. On the other hand, *B. thuringiensis* accumulates crystalline polypeptides that may be toxic to insects and is extensively used as biological insecticide for crop protection[3]. Although the pathogenesis and ecological characteristics may be different among the members of the *B. cereus* group, which is comprised of *B. thuringiensis*, *B. anthracis*, *B. cereus*, and *B. mycoides*, they differ in their 16S rRNA sequence by only nine nucleotides and differentiation of these closely related species has been difficult, requiring many phenotypic tests and molecular identification methods for their distinction[4-6]. Therefore, rapid methods of identification of the *Bacillus* genus are needed. Besides the potential use of anthrax spores as biological weapons, the food industry requires a rapid test to differentiate between the crop biocide *B. thuringiensis* and food-borne pathogens such as *B. cereus*.

ATR-IR permits the chemically based discrimination of intact microbial cells and produces complex biochemical patterns or fingerprints that are reproducible and distinct for different bacteria[7]. The complex spectra reflect the total biochemical composition of the microorganism, with bands due to major cellular constituents[7,8]. ATR-IR spectroscopy[8,9] have been reported for the rapid classification and discrimination of bacterial strains.

OBJECTIVE

The objective of this study was to develop a rapid method for the differentiation, identification, and classification of *Bacillus* species at the strain level using infrared microspectroscopy and multivariate pattern-recognition techniques.

MATERIALS & METHODS



- ATR-IR spectra were collected using a Digilab Excalibur 3500GX from 4000 to 700 cm^{-1}.
- SIMCA multivariate class models were produced of mean-centered, 2nd derivative spectra.

RESULTS

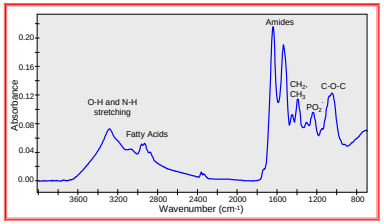


Figure 1. ATR-IR spectrum of *B. thuringiensis* ATCC 13366.

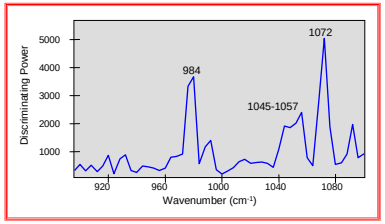


Figure 3. Discriminating power of the *B. cereus* family (using SIMCA).

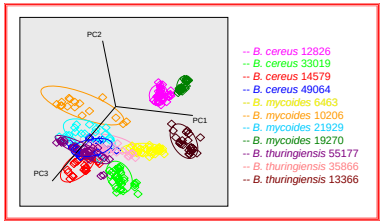


Figure 2. Class projections by SIMCA using second derivitized ATR-IR microscopy spectra (700-4000cm^{-1}) of selected *Bacillus* spp. on Ge.

	CS1@10	CS2@10	CS3@11	CS4@8	CS5@7	CS6@8	CS7@7	CS8@5	CS9@10	CS10@10	CS11@7
BC 12826	0.00										
BC 14579	48.66	0.00									
BC 33019	34.79	8.24	0.00								
BC 49064	45.64	10.00	12.12	0.00							
BM 10206	49.02	9.00	12.18	8.07	0.00						
BM 21929	46.89	10.02	7.53	6.47	7.42	0.00					
BT 13366	25.12	12.35	11.70	12.94	14.11	12.06	0.00				
BT 19270	15.52	36.86	30.26	34.68	40.11	37.32	18.36	0.00			
BT 35866	42.09	9.46	10.24	4.35	7.18	5.44	12.00	32.27	0.00		
BT 55177	52.41	9.60	8.63	8.95	9.62	7.77	13.45	39.11	8.38	0.00	
BT 55177	27.09	10.95	10.78	7.12	9.60	8.54	10.26	20.92	4.49	7.69	0.00

Table 1. Interclass distances for the *B. cereus* group (using SIMCA).

DISCUSSION

Figure 1 illustrates IR signal bands associated with *Bacillus* functional groups[7,8]. The fingerprint region of the spectra is a very reproducible and robust region that provides information on the total biochemical structure of the intact bacterial cell. Second derivatized transformations of the spectra removed baseline shifts and resolved overlapping bands, reducing variability[9]. Multivariate analysis, specifically Soft Independent Modeling of Class Analogy (SIMCA) used Principle Component Analysis (PCA) to build a modeling system of the transformed spectra. SIMCA classification exhibited clustering which permitted accurate differentiation of *Bacillus* at strain-level (Fig. 2) with zero misclassifications of *Bacillus* samples. Major discrimination occurred between 980-1075 cm^{-1} with major peaks at 984, 1045-1057, and 1072 cm^{-1} (Fig. 3). Major discrimination is presumably related to signal from lipids present in the cellular membrane. Table 1 shows interclass distances for the SIMCA model of the *B. cereus* group. The values represent Mahalanobis distances between the bacterial classes. Values greater than three have highly different dimensional clustering and are thought to group separately[7].

CONCLUSION

- Combining ATR-IR microspectroscopy with HGM's has shown promise as a high-throughput, rapid, reagent-free procedure to complement elaborate molecular identification methods for members of the *Bacillus* genus.
- Combining ATR-IR microspectroscopy with multivariate analysis could allow for development of a predictive modeling system.
- This could become a powerful tool for monitoring the safety of our food supply; including food spoilage, disease-transmission, and insect biocide activity caused by the *Bacillus* species.
- Generation of a library of major food-borne pathogens is needed for this approach to become a standard typing tool.

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REFERENCES

1-Jaquette CB, LR Beuchat. 1998. *J of Fd Prot* 61.1629-1635.; **2**-Christiansson A, J Bertilsson, B Svensson. 1999. *J of Dairy Sci* 82.305–314.; **3**-Agaisse H, L Didier. 1995. *J of Bact* 177(21).6027-6032.; **4**-Ash C, JA Farrow, M Dorsch, E Stackebrandt, MD Collins. 1991. *Int J of Sys Bact* 41.343-346.; **5**-Kim W, YP Hong, JH Yoo, WB Lee, CS Choi, SI Chung. 2001. *FEMS Micro Letters* 207.21-27.; **6**-Harell LJ, GL Andersen, KH Wilson. 1995. *J of Clinical Micro* 33.1847-1850.; **7**-Naumann D, CP Schultz, D Helm. 1996. *Infrared Spec of Biomol* ed. by [H.H. Mantsch and D. Chapman] 279-310.; **8**-Helm D, D Naumann. 1995. *FEMS Micro Letters* 126.75-80. **9**-Kansiz M, P Heraud, B Wood, F Burden, J Beardall, D McNaughton. 1999. *Phytochem* 52.407-417. **10**-Filip Z, S Herrmann, J Kubat. 2004. *Micro Research* 159.257-262.

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